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A non-apoptotic role for Fas/FasL in erythropoiesis

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ABSTRACT

Issues remain to be elucidated in the developmental regulation of erythropoiesis. In particular the role of Fas, a member of the tumor necrosis factor family of receptors despite much work remains unclear. During erythropoiesis, Fas is expressed at low levels on erythroblasts. For most cell types, Fas to FasL interaction causes apoptotic cell death via caspase activation. Here, we show that in humans, early erythroid progenitors are refractory to apoptosis triggered through Fas. Further during early human erythropoiesis, Fas triggered caspase activation provides a positive stimulus for erythroid maturation, and does not alter cellular proliferation or trigger apoptosis.

1. Introduction

Erythropoesis is maintained in part by negative regulation involving the so-called 'death receptor' Fas/FasL [1–4]. Early erythroid progenitors such as the $CD34^+$ erythroid burst forming units (BFU-E) express very low levels of Fas and are resistant to Fas induced apoptosis [5–7]. Upon further development however, cell surface Fas expression increases in early erythroblasts and they become sensitive to Fas-induced apoptosis [1,8]. Partial protection is provided by high levels of erythropoetin (Epo) upregulating GATA-1, which induces expression of the anti-apoptotic Bcl-X_L [8–10].

However, recent evidence indicates that Fas mediated caspase activation can positively regulate erythropoiesis. Research has shown that disruption of caspase-3 function in early erythroid progenitors inhibits erythropoiesis, with arrest at the pronormoblast stage [11–14]. Work has shown that heat shock protein-70 (Hsp-70) controls erythropoesis by blocking GATA-1 cleavage by caspase-3 [14]. Here we show for the first time that both caspase-8 and caspase-9 participate in human erythropoiesis in culture. Furthermore, we demonstrate that Fas and FasL, but not TNF-related apoptosis-inducing ligand (TRAIL), provide a positive stimulus for the maturation of early human erythroid progenitors.

2. Materials and methods

2.1. siRNA and antibodies

Control siRNA against GFP and siRNA to caspase-3 were previously described [11]. Other siRNAs include Fas (GUGCAGAUGUAAACCAAACTT), caspase-8 (CAACAAUAACACUGUCUCCTT) caspase-9 (ACACCCAGACCAGUGGACATT) (all from Memorial Sloan-Kettering Cancer Center). Fas (Ab-1) and FasL (Ab-1) and the agonistic Fas (Ab-2) antibodies came from Oncogene Research Products (La Jolla, CA). Caspase-3, -8 and -9 antibodies were from Santa Cruz Biotech (Santa Cruz, CA). Annexin V antibody was produced by Trevigen (Gaithersburg, MD). Antibodies to CD34, CD36 and Glycophorin-A (GPA) and fluorescent secondary antibodies came from Pharmingen (Heidelberg, Germany).

2.2. Realtime Polymerase Chain Reaction (rtPCR)

rtPCR was carried out as per the maker's instructions (Qiagen).

Abbreviations: caspases, cysteine–aspartic acid proteases; BFU-E, burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; Hsp-70, heat shock protein-70; FasL, Fas ligand; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; rtPCR, realtime polymerase chain reaction.

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2.3. Cell culture

Human hematopoietic progenitors were isolated and cultured as described [11]. Briefly, $CD34^+$ cells were cultured as follows: days 0–3, 10 ng/ml interleukin-3 (IL-3), 10 ng/ml stem cell factor (SCF); days 3–7, 10 ng/ml IL-3, 10 ng/ml SCF and 2 U/ml Epo; days 7–9, 10 ng/ml SCF and 2 U/ml Epo and after day 9, 2 U/ml Epo.

2.4. Transfection

$CD34^+$ cells were electroporated using a Nucleofector and a human CD34 cell nucleofector kit (Amaxa Biosystems, Koeln, Germany) as described [11].

2.5. Cell analysis

For assay of Fas or FasL by ELISA, either the Fas Ligand ELISA Kit or the Fas/APO-1 ELISA Kit was used (both from Oncogene Research Products, La Jolla, CA). Analysis of cell proliferation and morphology were as described previously [11].

2.6. Caspase activity monitoring

Caspase-3 activity was assayed using the ENZCHEK caspase-3 assay kit with a DEVD-AMC substrate (Molecular Probes, Eugene, OR) as previously described [11]. Caspase-8 activity was measured using the CASP8C caspase-8 assay kit with a Ac-IETD-pNa (Sigma) as per instructions. Caspase-9 activity was assayed using the Caspase-9 colormetric assay kit with LEHD-pNa (Biovision) as per instructions.

2.7. Apoptosis measurement

Apoptosis was assayed for using the TACS Annexin 5 Kit (TREVIGEN, Gaithersburg, MD) as per the manufacturer's instructions. CD34 cells grown for 7–9 days and then washed and cultured without cytokines for 6 h provided the positive control. Terminal deoxynucleotidyl transferase was assayed using the ApoDirect DNA Fragmentation Assay Kit (BioVision).

2.8. FACS analysis

Facs analysis was carried out as reported previously [11].

3. Results

We have previously demonstrated that isolated human $CD34^+$ progenitor cells maintained with the appropriate regimen of cytokines constitute a valid human erythropoiesis model. Normal developmental stages occur with a large fraction of progenitor cells (>30%) becoming enucleated reticulocytes, another 30% becoming orthochromatic normoblasts and the rest are erythroblasts (Fig. 1A) [11]. We used this system to investigate whether caspase-8 and -9, in addition to caspase-3, are utilized during erythropoiesis.

To determine if caspase-3 activity was triggered via caspase-8 or-9 $CD34^+$ progenitors were transfected with siRNA to inhibit caspase-8, -9, or –3 on day 0, then cultured and monitored for caspase activity over time (Fig. 1B). In controls, the caspase activity for all three caspases are high at day 0 in undifferentiated $CD34^+$ cells and then rises to a peak at day 3 prior to declining for the rest of the culture (Fig. 1B). Caspase-3 siRNA treatment reduced peak caspase-3 activity by approximately 50% between days 0 and 10, consistent with the approximate 50% siRNA transfection efficiency in these cells [11]. Caspase-3 activity was also reduced by 50% with siRNA to caspase-8 and Fas but with siRNA to caspase-9 the reduction was only 25%. Further, caspase-8 activity was only reduced by siRNA to caspase-8 and Fas. In contrast caspase-9 activity was reduced by caspase-8, -9, and Fas but not with caspase-3 siRNA. (Fig. 1B). Hence, caspase-8 and Fas are required for both caspase-9 and -3 activation.

SiRNAs knockdown efficiency was confirmed by realtime PCR, performed 24 h after nucleofection. The 50–60% reduction in mRNA level is in agreement with this systems previously reported transfection efficiency (Fig. 1C).

This raised the question how is Fas expression affected by Fas siRNA [5–7]. To answer this we treated cells with Fas siRNA. Fas-specific siRNA further reduced Fas's normally low expression levels on days 0–4. Also normal Fas expression increases observed by day 5 were delayed and peak Fas expression (day 8) was decreased by 30% (Fig. 2A). However, Fas expression caught up to and exceeded the control by day 11, possibly due to siRNA dilution and increased Fas production as the cells proliferate.

Reticulocyte enucleation was used to judge the effect of siRNA treatment on erythropoiesis (Fig. 2B). Reduced enucleation, comparable to that seen in caspase-3 activity (Fig. 1B), was found with each of the caspase and Fas siRNA treatments.

Decreasing erythroblast enucleation suggested that reduced caspase activity blocks erythroid maturation. Morphological analysis was used to assess at which developmental stage the block occurs (Fig. 2C). All cultures are identical on day 7. Control cultures developed into late erythroblasts with a few residual basophilic blasts. In siRNA-treated cultures, pronormoblasts increase to constitute nearly half of the day 17 culture. Late erythroblasts numbers were also reduced 50%. These results suggest a developmental block at the pronormoblast stage. This is supported by FACS analysis (Fig. 2D). The early progenitor marker CD34 declined in all cultures, as expected. CD36 expression, however, declined after day 7 in control cultures but it remained high in siRNA treated cultures. Glycophorin A expression increased in controls, reaching ca. 95% positive cells by day 17, whereas siRNA treated cells plateaued at 60% positive cells. Hence, CD36 and glycophorin A expression patterns suggest siRNA treatment retards culture development.

This suggests that caspase activation is required for erythroid maturation prior to culture day 7. To test this, Fas siRNA treatment was applied on day 0, 3 or 7 of culture (Fig. 2E). Maximal erythroid developmental inhibition required treatment with Fas siRNA on day 0. Fas siRNAs effect was diminished at day 3 and, not significant on day 7, consistent with proerythroblasts caspase activation.

To confirm that caspase-induced apoptosis of erythroid progenitors was not involved, we monitored the cultures by Annexin-V staining. Control and siRNA-treated cultures exhibited no differences in apoptosis (Fig. 3A). Hence apoptosis was not responsible for the differences observed between control and siRNA-treated cultures. This work has confirmed by FACs analysis to monitor DNA fragmentation using Terminal Deoxynucleotidyl Transferase (Fig. 3B). Again the control and siRNA treated cultures had no differences in the detectable level of DNA fragmentation. We further assessed the effect of Fas siRNA on the ability of the cells to form colonies in semi-solid medium. Fas siRNA did not altered the observed numbers of BFU-Es, colony forming unit-erythroids (CFU-Es), or CFU-GEMMs (granulocyte erythroid monocyte megakaryocytes) present (Fig. 3C). We used agonistic antibody (Fig. 3D) and different concentrations of FasL (Fig. 3E) to explore the cells susceptibility to apoptosis triggered by Fas. In both cases, cells at day 0 were entirely resistant to apoptosis.

During erythropoesis susceptibility to Fas-triggered apoptosis rose in parallel with levels of Fas (compare Fig. 3D and E with 3F), as previously reported [1,2]. We determined the peak levels of cell-associated and soluble FasL occurred at day 2 (Fig. 3F),

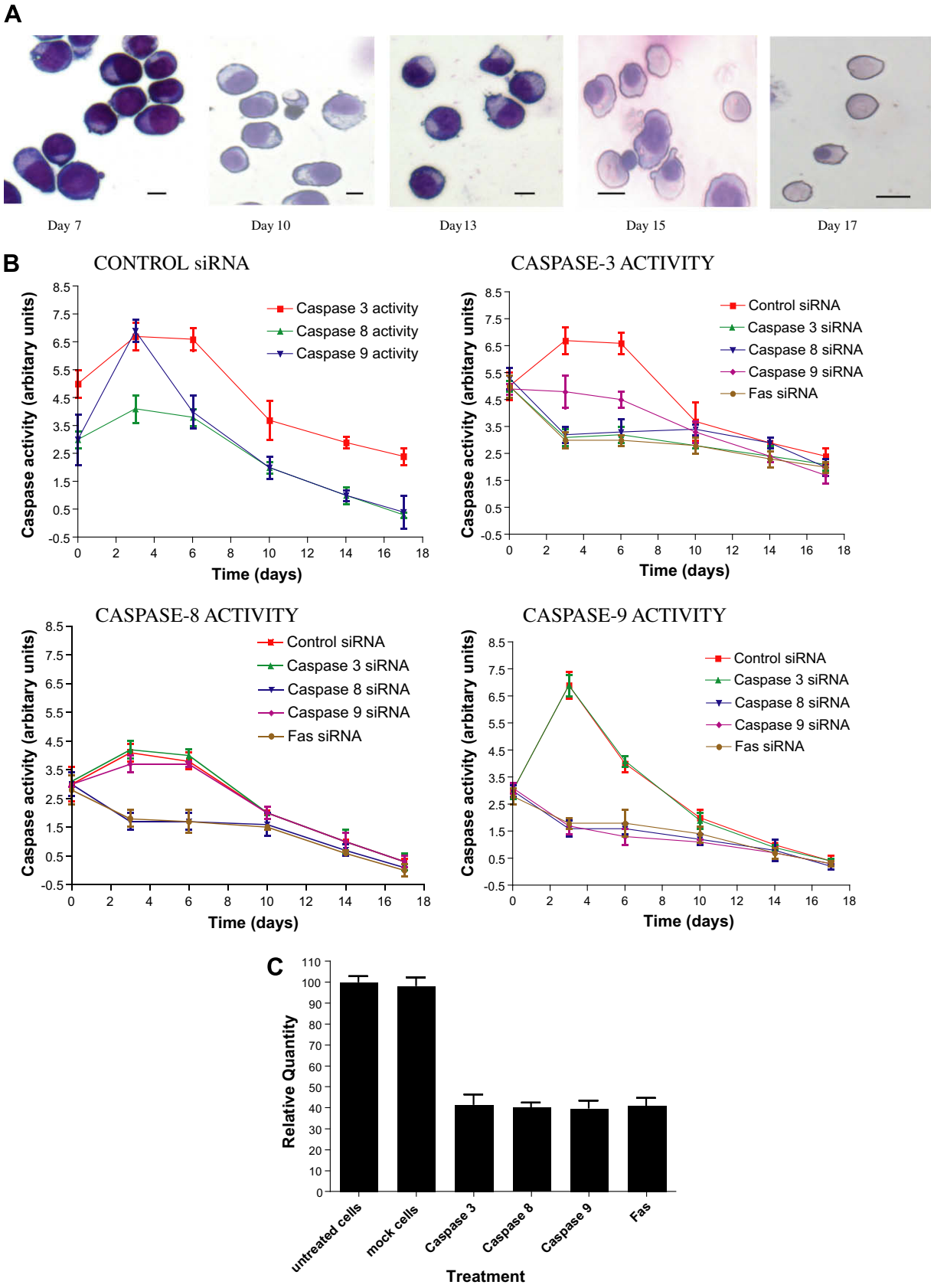

Fig. 1. siRNA to caspase enzymes are effective in early erythroblasts. (A) Cells stained with Wright-Giemsa showing pronormoblasts (day 7), pronormoblasts and basophilic normoblasts (day10), basophilic normoblasts (day 13), orthochromatic normoblasts and enucleated reticulocytes (day15) enucleating, and enucleated reticulocytes and orthochromatic normoblasts (day17). Scale bar 5 μm. (B) Purified, human $CD34^{+}$ cells were transfected at day 0 with siRNA to GFP (Control) or the indicated caspase or Fas and cultured [11]. Caspase enzyme activity was assayed over time. (C) Representative rtPCR to test the relative efficiency of the various siRNAs used. The above are representative of three or more experiments.

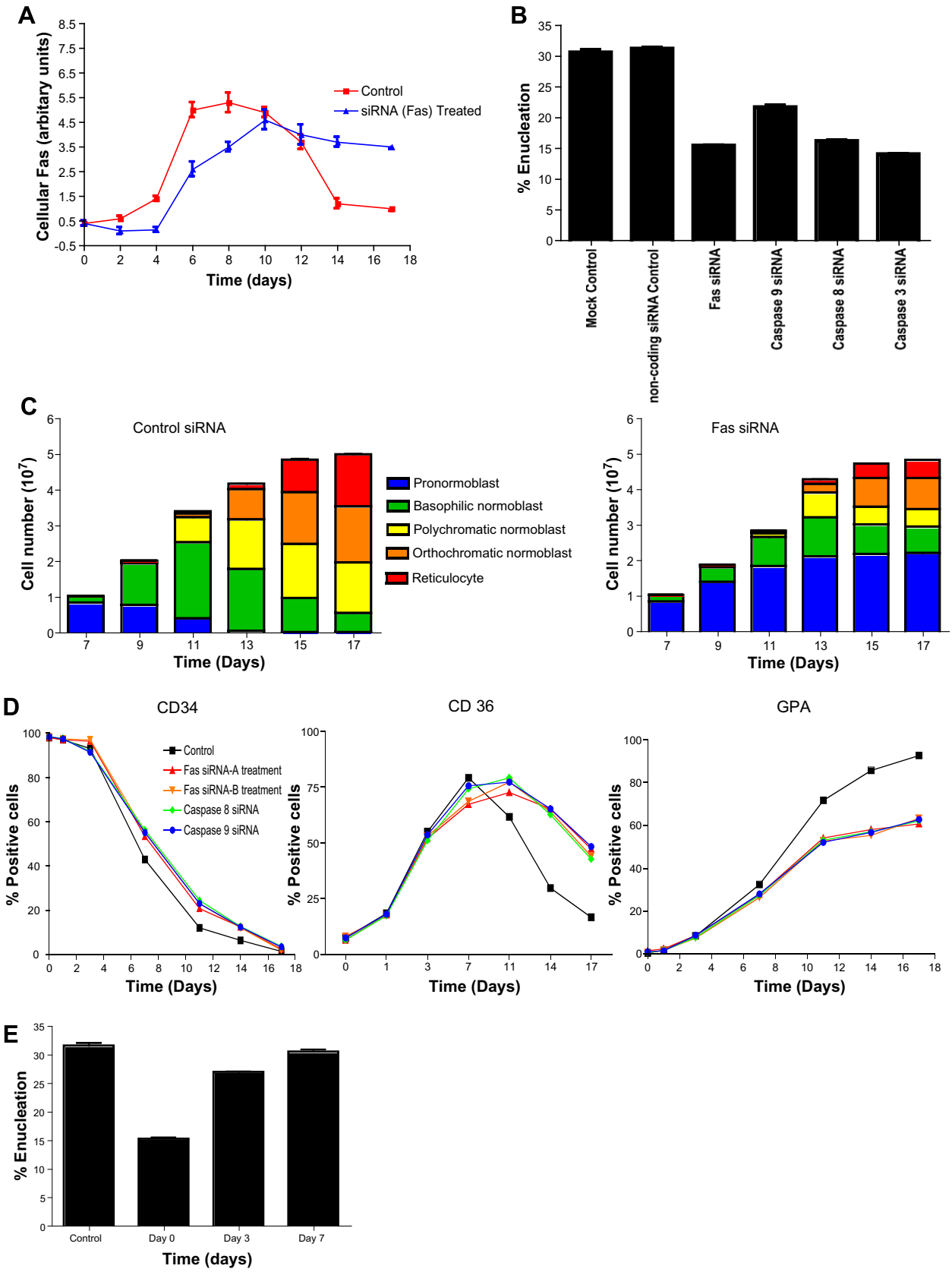

Fig. 2. siRNA to caspase enzymes and to Fas block erythroid maturation at the pronormoblast stage. $CD34^+$ cultures were transfected with either control siRNA (GFP) or Fas siRNA (A) and Fas expression monitored. (B) Cultures were scored for enucleated cell percentage (day 17). (C) Cultures were evaluated for cellular morphology (D), and for cell surface marker expression. The above are representative of three or more experiments. (E) $CD34^+$ Cultures were treated with Fas or with GFP siRNA (Control). Percent enucleation was evaluated at day 17. Error bars indicate S.D. of cell counts from five fields of view per experiment and three experiments.

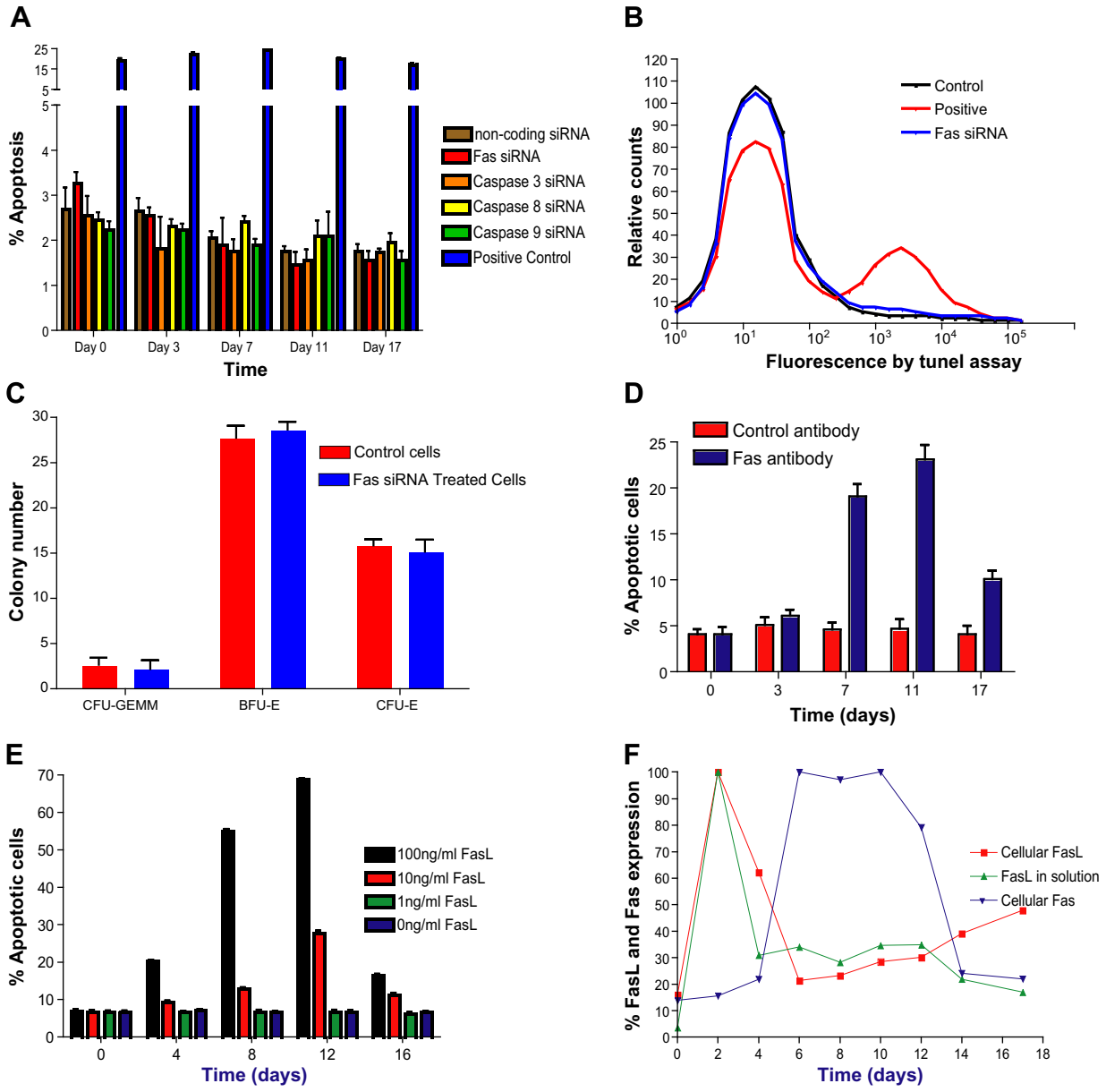

Fig. 3. Inhibition of caspase activity does not affect apoptosis and the levels of Fas and FasL change with time in culture. (A) $CD34^+$ cells were transfected with the indicated siRNAs at day 0 and evaluated for Annexin-V binding (% Apoptotic Cells). For the positive controls, 7-day erythroblast cultures placed in media without cytokines for 24 h. (B) $CD34^+$ cells were transfected with Fas siRNAs at day 0 and monitored 24 h later for DNA fragmentation by TdT. (C) $CD34^+$ cells were transfected with Fas siRNAs at day 0 and grown in semi-solid media. (D) $CD34^+$ cells were cultured as normal and challenged at various time points with agonistic antibody for 12 h before being assayed for Annexin-V binding. (E) $CD34^+$ cells were cultured and challenged for 24 h at various time points with varying concentrations of soluble FasL and monitored for Annexin-V binding. (F) Fas and FasL levels on cells or in the media were assayed by ELISA and expressed as a percentage of the maximum observed expression. Maximum soluble FasL concentration observed was 1.5 ng/ml.

when Fas expression is low. To test whether soluble FasL stimulates erythropoiesis, we depleted the media of FasL by washing the cells at 2 day intervals. This decreased the soluble FasL (Fig. 4A) and retarded cellular development, with ca. 20% of the culture blocked at the pronormoblast stage (Fig. 4C), resulting in significantly reduced enucleation at day 17 (Fig. 4B). The failure to completely block development by media depletion may be due to residual FasL that accumulated over the 2 days of incubation or to FasL present on the erythroblast surface. FasL depletion did not alter cellular proliferation (Fig. 4C) or apoptosis (data not shown). Addition of 1 ng/ml recombinant FasL, restored cultures to nearly normal erythroid maturation (Fig. 4B and C). Other death receptors on the surface of erythroid progenitor cells include DR4 and DR5, which bind the ligand, TRAIL [1,15]. However, TRAIL addition to depleted cultures did not rescue erythroid maturation (Fig. 4D). Although we cannot exclude some redundancy in the death receptors and the caspases that can fulfill the requirement, clearly Fas/FasL is sufficient to stimulate early erythroid development.

4. Discussion

Caspase-3. and caspase-7 activation early in erythropoiesis is well documented and in mice Fas had been identified as being involved, but if this occurred in the human system was unclear [3,12]. Using siRNA on our ex vivo human $CD34^+$ erythroid

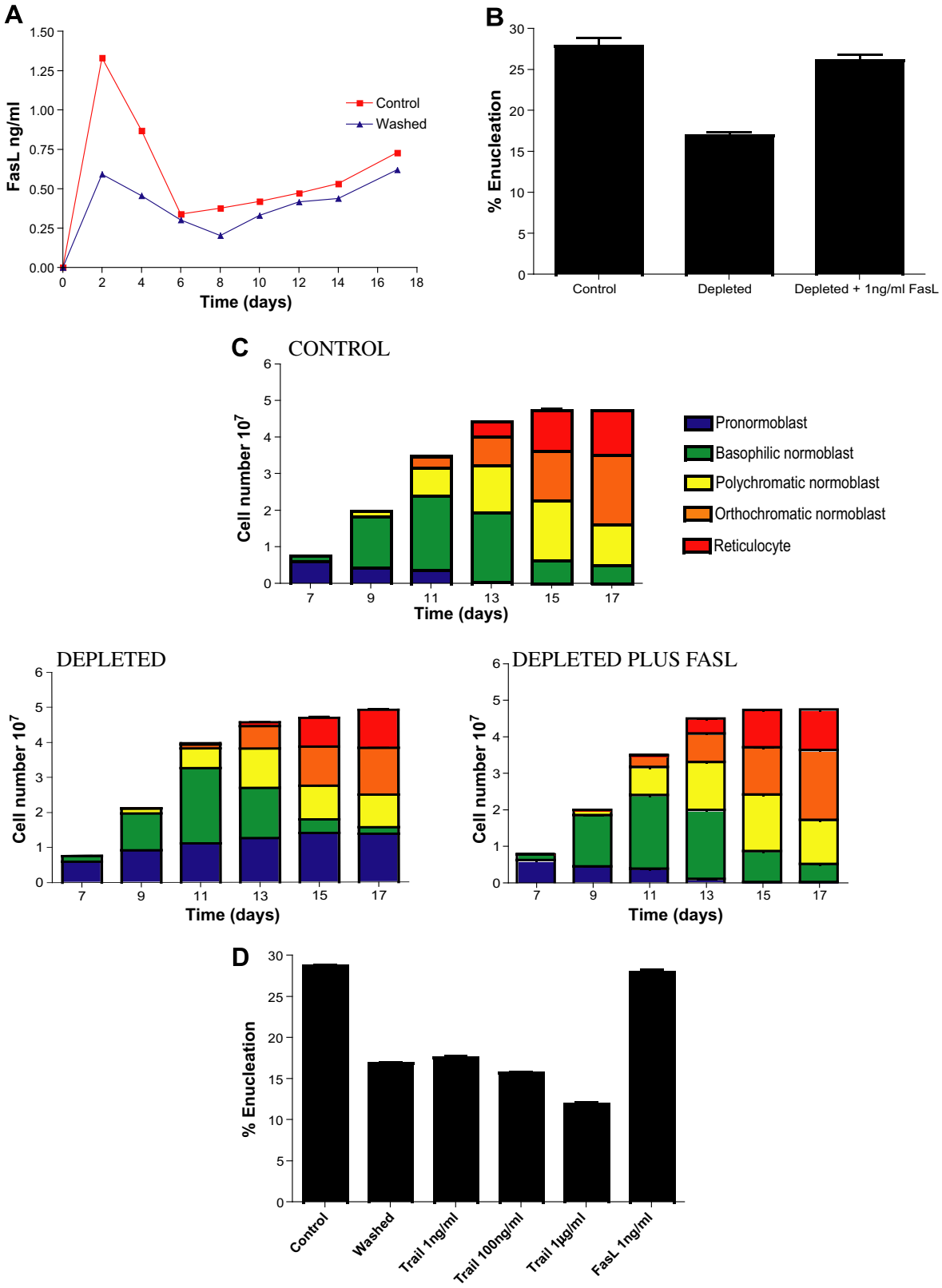

Fig. 4. Developmental block produced by media depletion is rescued by FasL addition but not TRAIL. CD34$^+$ cultures were depleted of FasL by washing and resuspending in fresh media every 2 days. Control cells were resuspended in normal media. (A) FasL was measured by ELISA. Cultures were evaluated for percent enucleation (day 17) (B) and over time for cellular morphology (C) in parts B and C 1 ng/ml FasL was added after washing to rescue erythroid differentiation. (D) CD34$^+$ cultures were treated as described for A. except for the addition of the indicated supplements. Cultures were evaluated for percent enucleation (day 17).

progenitor cells, we selectively reduced expression of the Fas pathway to test its involvement in erythroid maturation. Here, we show that caspase activation via Fas/FasL has a stimulatory effect on erythroid maturation.

Without caspase activation, erythroblasts do not develop beyond the pronormoblast stage, a transition that begins around culture day 7. However, the pronormoblast block is most effectively produced by reducing caspase-3 [11] or Fas (Fig. 2) expression before day 3. At this point, erythroblasts are refractory to apoptosis, possibly because Fas expression is low. Nevertheless, soluble FasL engages cell surface Fas and triggers caspase activation because removal of either FasL (by washing) or Fas (using siRNA) reduces caspase activation and blocks erythroid maturation. Notably, autocrine cytokine depletion can be rescued by FasL but not TRAIL, another erythroid death receptor ligand.

Studies have reported that Fas/FasL induces erythroblast apoptosis. Erythroid homeostasis relies on cytokines including Epo promoting cell survival by inducing the anti-apoptotic Bcl-2 family proteins [7–10,14,16]. However, FasL expressed on late erythroblasts induced apoptosis [1,2]. deMaria found that for Fas-sensitive erythroblasts two factors decided cell fate; Fas stimulation levels and Epo concentration for culture maintainence [1]. Sublethal doses of anti-Fas antibody caused caspase activation and GATA-1 cleavage, but not apoptosis. However, recent work showed that HSP-70 blocks caspase-3 mediated cleavage of GATA-1 during erythropoiesis [14]. Nevertheless, culture maturation was inhibited at the basophilic erythroblast stage. This contrasts with our work and may be due to the early developmental stage at which we tested our cultures. Fas/caspase depletion only restricts development in the first days of culturing, corresponding to BFU-E and CFU-E progenitors. The up-regulation of expression of Fas and FasL sensitizing early human erythroblasts to apoptosis has only been shown with high interferon levels [17].

Studies in mouse models have suggested that Fas and FasL play a negative role in the erythroid regulation. However, our human erythroblast studies, demonstrate a positive function for the Fas/caspase signaling pathway in erythropoesis. The reason for this difference is unclear but may be another example of the difference in species [18].

Caspase mediated apoptosis is central for organism development [19]. However, several instances have been identified where caspase activation without apoptosis is implicated in lineage specific differentiation. Studies using caspase inhibitors blocked ex vivo differentiation of lens epithelial cells [20], keratinocytes [21], megakaryocytes [22] and erythroblasts [11]. We have demonstrated that caspase activation via Fas/FasL stimulation is required for erythroid maturation. Furthermore, the stimulatory activity of caspases in erythroid maturation is transitory and specific for $CD34^+$ early erythroid progenitors, despite the low levels of Fas expressed on these cells. Given that Fas is expressed at low levels on many different cell types throughout development, it may be that non-apoptotic caspase activation has a much broader role in human development than is currently recognized.

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